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Mouse Oocyte Mitogenic Activity Is Developmentally Coordinated throughout Folliculogenesis and Meiotic Maturation

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Oocytes secrete soluble factors that regulate the growth and differentiation of follicular cells, including maintenance of the distinctive cumulus cell phenotype. This study determines whether the mitogenic activity of oocytes is developmentally regulated and examines the responsiveness of follicular cells to oocytes at different stages of follicular development. Prepubertal SV129 mice of varying ages were primed with 5 IU equine chorionic gonadotropin (eCG) and oocytes/zygotes collected either 46 h post-eCG (immature oocytes), 12 h after administration of 5 IU human CG (hCG; ovulated ova), or 12 h post-hCG and mating (zygotes). Mural granulosa cells (MGC) from antral follicles and GC from preantral follicles were cultured ± denuded oocytes (DO) for 18 h, followed by a 6-h pulse of [^{3}H]thymidine as an indicator of cellular DNA synthesis. Coculturing MGC with meiotically maturing oocytes led to a dose-dependent increase in [^{3}H]thymidine incorporation (20-fold above control levels at 0.5 DO/μl). However, [^{3}H] counts remained unchanged from control levels when cultured with meiotically incompetent DO from 11- to 15-day-old mice (3% germinal vesicle breakdown; GVB), irrespective of dose of DO or developmental status of GC (MGC or preantral GC). In some treatments, spontaneous meiotic resumption of competent oocytes was prevented by culturing with 5 μM milrinone, a selective inhibitor of oocyte-specific cyclic nucleotide phosphodiesterase. The mitogenic capacity of oocytes was found to decline during and after oocyte maturation. [^{3}H]Thymidine incorporation in MGC was highest (11-fold above controls) when cultured with meiotically inhibited (milrinone-treated) GV DO, stimulated 5.5-fold by culture with maturing oocytes, 3-fold with ovulated ova, and unstimulated by zygotes. [^{3}H]Thymidine incorporation in MGC was not altered by the dose of milrinone, either in the presence or absence of DO. Metaphase I marked the beginning of the decline in the capacity of oocytes to promote MGC DNA synthesis. These results demonstrate that the capacity of oocytes to promote proliferation of granulosa cells follows a developmental program, closely linked to oocyte meiotic status, increasing with the acquisition of meiotic competence and declining during and after oocyte maturation. © 2001 Academic Press

Key Words: **oocyte mitogen; oocyte meiotic competence; folliculogenesis; granulosa cell; oocyte maturation.**

INTRODUCTION

Oocyte–follicular cell interaction is a bidirectional process: not only does the follicular environment determine to a large extent the developmental capacity of the oocyte, but also paracrine signals from the oocyte play a critical role in the growth and development of the follicular compartment. Follicle growth and development fail in the absence of the oocyte, whether lacking due to genetic deficiencies or experimental ablation. Oocytes were first implicated in the regulation of follicular cells by findings that surgical removal of oocytes from preovulatory rabbit follicles causes spontaneous luteinization (el-Fouly *et al.*, 1970). It is now known that oocyte-derived factors modulate a broad range of follicular cell functions, including: cumulus cell mucification and hyaluronic acid production (Buccione *et al.*, 1990; Salustri *et al.*, 1990b); cellular proliferation (Vanderhyden *et al.*, 1992; Lanuza *et al.*, 1998; Li *et al.*, 2000); steroid (Vanderhyden *et al.*, 1993) and inhibin synthesis (Lanuza *et al.*, 1999); and mRNA expression of urokinase-type plasminogen activator (uPA; Canipari *et al.*, 1995), luteinizing hormone receptor (LHR; Eppig *et al.*, 1997), and kit-ligand (KL; Joyce *et al.*, 1999). Despite considerable

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efforts to characterize these oocyte-derived factor(s), their true identity remains elusive. To date, the best candidates are members of the transforming growth factor-β (TGFβ) superfamily; including TGFβ1 (Salustri *et al.*, 1990a), growth-differentiation factor-9 (GDF-9; Elvin *et al.*, 1999), and bone morphogenic protein-15 (BMP-15; Otsuka *et al.*, 2000), as these oocyte-secreted proteins are able to mimic many of the effects of oocytes on follicular cells.

Two of these identified oocyte-derived growth factors are essential for normal follicle development as GDF-9-deficient female mice (Dong *et al.*, 1996) and BMP-15-deficient ewes (Galloway *et al.*, 2000) are infertile due to a complete block in folliculogenesis at the primary follicle stage. These animals exhibit remarkably similar ovarian phenotypes, characterised by small "streak ovaries" containing a multitude of primordial and primary follicles and also tumor-like, oocyte-free granulosa cell nests (Dong *et al.*, 1996; Juengel *et al.*, 2000). Interestingly, while ewes homozygous for the Inverdale gene (which contains the mutation in the BMP-15 gene) are infertile, heterozygotes have an increased ovulation rate compared to wild-type ewes. Hence, while absence of oocyte GDF-9 or BMP-15 cause dramatically altered gene expression in follicular cells leading to perturbed folliculogenesis and sterility, dosage of oocyte BMP-15 affects fecundity in fertile animals.

The capacity of oocytes to alter granulosa cell functions is developmentally regulated, depending on the stage of oocyte growth and maturation, the developmental state of the granulosa cells, and the oocyte factor in question. In general terms, growing meiotically incompetent oocytes are less effective in regulating follicular cell functions than fully grown oocytes, even though growing oocytes have been shown to contain GDF-9 (Elvin *et al.*, 1999; Jaatinen *et al.*, 1999) and BMP-15 proteins (Otsuka *et al.*, 2000). For example, growing mouse oocytes do not produce cumulus expansion-enabling factor (CEEF) and are not able to suppress uPA or KL synthesis (Vanderhyden *et al.*, 1990; Canipari *et al.*, 1995; Joyce *et al.*, 2000), but are able to regulate steroidogenesis and suppress LHR mRNA expression (Eppig *et al.*, 1997; Vanderhyden and Macdonald, 1998). Oocyte CEEF, uPA-suppressor, and KL-suppressor activities are acquired as oocytes achieve meiotic competence, and are maintained throughout antral development and during meiotic maturation, but are lost by the zygotic stage. Oocytes are also able to promote the proliferation of follicular granulosa cells (Vanderhyden *et al.*, 1992), although the developmental expression of this oocyte factor remains unclear. Previous studies indicate that fully grown, meiotically maturing oocytes secrete a potent mitogen to which all of the major granulosa cell phenotypes are able to respond, including relatively undifferentiated granulosa cells from preantral follicles, and differentiated mural granulosa cells and cumulus cells from antral follicles (Vanderhyden *et al.*, 1992; Lanuza *et al.*, 1998; Li *et al.*, 2000). It is unknown whether small, growing oocytes are major drivers of preantral follicle growth. It is also not known how oocyte mitogenic activity is regulated in relation to the acquisition of meiotic capacity, nuclear status of meiotically competent oocytes, or during early embryo development. Hence, the objectives of this study are to examine the developmental expression of oocyte mitogenic capacity throughout folliculogenesis and oocyte meiotic maturation. Evidence reported here demonstrates that the mitogenic capacity of oocytes is closely coupled to oocyte meiotic competence and maturation status. Meiotically competent, GV-stage oocytes exhibit the highest growth-promoting activity, greater than growing oocytes, meiotically maturing oocytes, or zygotes.

MATERIALS AND METHODS

Collection and Preparation of Granulosa Cells

Mice used in this study were maintained at The Queen Elizabeth Hospital on a 14:10-h light:dark cycle with pelleted food and water available ad libitum. This study was approved by local Animal Ethics Committees and was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Reproductive tracts were collected from immature SV129 mice of varying age depending on the experiment, primed with 5 IU of equine chorionic gonadotrophin (eCG; Folligon, Intervet, Castle Hill, Australia). Ovaries were dissected free of adherent adipose and connective tissues and placed in 25 mM Hepes-buffered tissue culture medium-199 (H-TCM; ICN, CA) supplemented with 2 mM Na pyruvate (Sigma, St Louis, MO), 100 IU/ml Penicillin G (Sigma), and 100 μg/ml streptomycin sulfate (Sigma) and 0.3 mg/ml polyvinyl alcohol (PVA; Sigma).

For the preparation of mural granulosa cells (MGC), ovaries were obtained from 23- to 32-day-old mice 46 h after eCG treatment. Large antral follicles in intact ovaries were punctured with a 27-gauge needle and MGC were gently squeezed from follicles. All debris (stromal and thecal tissue, preantral follicles, and naked oocytes) were carefully removed from the ovary puncture dishes, and the remaining MGC were collected by centrifugation. Cells were washed once in H-TCM and twice in culture media. Granulosa cells (GC) from small antral follicles and preantral follicles were obtained by collecting follicular cells from mice as young as 11 days old by using a similar procedure to that of the older mice. In general, these younger immature mice were also primed with 5IU eCG, although not in one experiment (see Table 2).

Preparation of Oocytes at Different Stages of Development

Immature cumulus–oocyte complexes (COC) were collected from punctured follicles at the same time as GC were collected. Only COC with a uniform covering of compacted cumulus cells and with a light and evenly pigmented ooplasm were used in this study. Denuded oocytes (DO) completely devoid of cumulus cells were generated by vortexing COC for ~2 min in H-TCM.

To determine at what stage of oocyte development an oocyte mitogen is produced, oocytes were collected from follicles at varying stages of development by collecting oocytes from mice of varying prepubertal age. The mitogenic capacity of oocytes with three differing degrees of meiotic competence were compared: (1) growing, meiotically incompetent oocytes from preantral follicles,

collected from 11- to 15-day-old mice; (2) growing oocytes with low meiotic competence from periantral follicles from 16- to 19-day-old mice, and; (3) fully grown, meiotically competent oocytes from antral follicles collected from 21- to 32-day-old mice (see Figs. 2 and 3, Table 2).

Meiotically competent oocytes were examined for their mitogenic capacity at varying stages of meiotic maturation, and also under conditions in which meiotic maturation was inhibited. Spontaneous meiotic resumption was artificially inhibited by collecting and culturing fully grown DO with 5 μM milrinone (Sigma), a potent inhibitor of oocyte-specific type-3 phophodiesterases (see Figs. 4 and 5).

Oocytes at different stages of maturation were generated by partially or completely maturing meiotically competent oocytes (either *in vivo* or *in vitro*), before addition to freshly collected MGC. To generate discrete groups of meiotically synchronized oocytes (see Fig. 6 for illustration of experimental design), four groups of 24- to 28-day-old mice were primed with eCG at 3 hourly intervals and then killed 46 h later. COC were collected and denuded and then the first group was allowed to spontaneously mature in culture medium (see below) in Nunc (Roskilda, Denmark) 4-well dishes for 9 h (treatment 1), the second group for 6 h (treatment 2), the third for 3 h (treatment 3), and the fourth for 0 h (treatment 4). The precultured oocytes, each group being at progressively advanced stages of meiosis, were then added to freshly collected MGC at a concentration of 50 DO/well. After 3 h of coculture, DO were removed from the wells and the remaining MGC were cultured for a further 21 h. This experimental design generated four treatment groups where MGC were exposed to oocytes in four different, nonoverlapping phases of meiosis.

Ovulated *in vivo* matured oocytes and zygotes were collected from oviducts of unmated or mated 23- to 32-day-old eCG-primed mice, respectively, administered 5 IU of hCG (Pregnyl; Organon, Sydney, Australia) 46 h after eCG treatment. Only ova with a polar body or zygotes with two pronuclei were used. These mature oocytes and zygotes were then added to freshly collected MGC and compared to *in vitro* maturing and GV-arrested oocytes in terms of their capacity to promote MGC DNA synthesis (see Fig. 4).

Culture Conditions and Experimental Set-Up

To determine cell numbers, a sample of GC was dissociated as previously described (Luciano *et al.*, 2000) and counted by using a haemocytometer. For culture, GC were not dissociated but rather cultured as clumps of cells (Luciano *et al.*, 2000). Granulosa cells were cultured at a density of approximately 25,000 GC/well in bicarbonate-buffered TCM supplemented with PVA, 0.23 mM Na pyruvate, and antibiotics (B-TCM) in 96-well plates (Falcon, Franklin Lakes, NJ). Depending on the individual experiments, DO/zygotes, hormones, reagents, and media were added to the wells in a known volume of medium prior to GC, giving a final volume of 125 μl after addition of GC (2×10^5 GC/ml). DO and GC were generally cocultured for 24 h (except in the one experiment where DO were added for just 3 h; see Fig. 6) at a concentration of 16 DO/well (0.128 DO/μl) unless otherwise stated. To produce oocyte-conditioned medium (OCM), meiotically competent DO were cultured in B-TCM for 24 h at 1.25 DO/μl in 96-well plates in the absence of GC. Within each experiment, all treatments were carried out in at least duplicate wells. Each experiment was replicated on 4–6 occasions utilising ~6–12 mice per experimental replicate. Cells were cultured in an atmosphere of 37°C, 96% humidity in 5% CO_2 in air for 18 h, followed by a further 6-h pulse of 15.4 kBq tritiated thymidine ([^{3}H]thymidine; ICN).

Assessment of Oocytes and Cell Cultures

During or at the completion of culture, oocytes/zygotes were visually assessed for developmental progression under an inverted light microscope. Oocytes were classified as: immature (possessing an intact germinal vesicle, GV), germinal vesicle breakdown (GVB), metaphase II (MII; presence of a polar body, PB), fertilized (2 pronuclei present, 2PN), or as 2-cell embryos. At the completion of culture, 80 μl of the culture media was collected and frozen (−20°C) for steroid analysis. Culture supernatants were assayed for progesterone content by using a radioimmunoassay kit (Diagnostic Systems Laboratories, Webster, TX) according to the manufacturer's instructions. The kit, which utilises an ^{125}I-labelled progesterone tracer, has a sensitivity of 0.25 pmol/ml and an intra-assay coefficient of variability of 5.4%. Following culture, GC were harvested by using a Tomtec Harvester 96 onto a filtermat and incorporated [^{3}H]thymidine was quantified by using a Wallac microbeta scintillation counter. [^{3}H] counts were used as a measure of incorporation of [^{3}H]thymidine into follicular cell DNA and as an indicator of the proportion of cells in S-phase, hence providing an indication of the level of GC DNA synthesis. In such experiments, GC [^{3}H]thymidine uptake does not necessarily afford a precise measure of cell proliferation, but rather is a reflection of short-term cellular events that may be correlated with rates of cell division. Previous studies have demonstrated that [^{3}H]thymidine uptake by GC is associated with an increase in total GC DNA content when stimulated by oocytes (Lanuza *et al.*, 1998), or in cell numbers when stimulated by IGF-I in longer term cultures (Di Blasio *et al.*, 1994).

Statistcal Analysis

Within each experimental replicate, GC [^{3}H]thymidine incorporation data were expressed as a fraction of the control treatment to reduce between replicate variance. Data from GC [^{3}H]thymidine incorporation were examined using either one-way or two-way ANOVAs and differences between treatment means tested by using Tukey–Kramer post hoc comparisons. Differences in the proportion of oocytes undergoing GVB (see Fig. 2) were examined by using Chi-squared analysis.

RESULTS

Response of MGC to Denuded Oocytes and Oocyte-Conditioned Medium

Coculturing MGC with DO increased MGC [^{3}H]thymidine incorporation in a dose-dependent manner (Fig. 1A). As few as 8 DO/125 μl well (0.064 DO/μl) led to a 2.5-fold increase in MGC [^{3}H] incorporation above control levels ($P < 0.01$) and at 62 DO/well levels were 16-fold higher than controls. In the absence of DO, IGF-I caused a 1.7-fold increase in MGC [^{3}H] incorporation ($P < 0.05$). DO also caused a dose-dependent increase in MGC DNA synthesis in the presence of IGF-I (Fig. 1). Coculturing DO with MGC also led to a dose-dependent increase in progesterone secretion by MGC but only to levels 1.9-fold above control levels

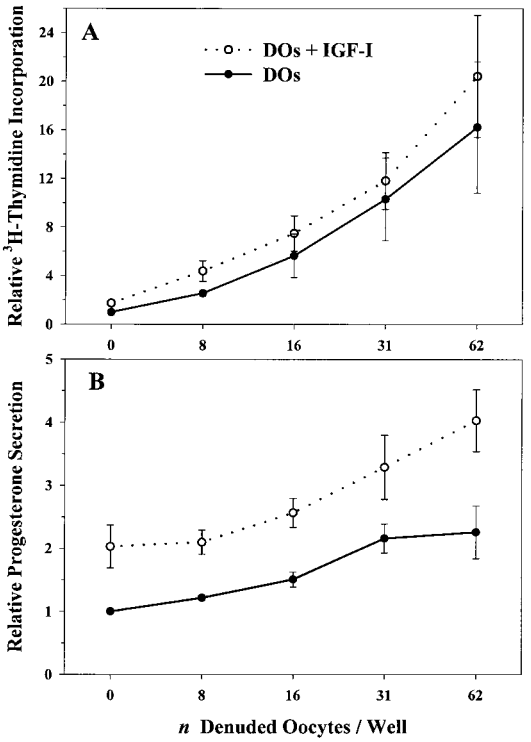

FIG. 1. Dose response of denuded oocytes (DO) on mural granulosa cell DNA synthesis (A) and progesterone secretion (B). MGC were cultured with increasing numbers of meiotically competent DO either alone or together with 50 ng/ml IGF-I. Points represent relative mean values ± SEM expressed as a fraction of the control (MGC in the absence of IGF-I and DO) from five replicate experiments. There was no IGF-I × DO interaction ($P > 0.05$; two-way ANOVA).

(Fig. 1B). In contrast, DO suppressed MGC progesterone secretion when cultured in the presence of FSH (data not shown), as previously reported (Vanderhyden *et al.*, 1993). Progesterone production was doubled by IGF-I alone and increased 4-fold above control levels in the combined presence of IGF-I and 62 DO/well. The effects of DO and IGF-I on MGC progesterone levels and DNA synthesis were additive as no IGF-I × DO interaction was observed for either variable ($P > 0.05$; two-way ANOVA).

Oocyte-conditioned medium (OCM), whether fresh (Table 1) or frozen (data not shown), failed to alter the incorporation of [^{3}H]thymidine into MGC. In contrast, coculture with DO at an equivalent concentration significantly ($P < 0.001$) increased [^{3}H] incorporation above control levels. The failure of OCM to increase MGC [^{3}H] incorporation was also evident when oocyte concentrations were increased to 62 DO/well (0.5 DO/μl), which leads to a 16-fold increase when oocytes are cocultured (Fig. 1). It has previously been shown that OCM is ineffective in inducing proliferation (Lanuza *et al.*, 1998) or suppressing LH receptor expression (Eppig *et al.*, 1997), although the steroid-regulating capacity of oocytes is retained (Vanderhyden *et al.*, 1993).

Growth-Promoting Activity of Meiotically Incompetent Oocytes

To examine whether meiotically incompetent oocytes were able to promote granulosa cell DNA synthesis, oocytes were collected from young prepubertal mice at an age when follicular antrum development is incomplete. Ovaries from 11- to 15-day-old mice have predominantly preantral follicles containing growing, meiotically incompetent oocytes (3.4% spontaneous germinal vesicle breakdown [GVB] *in vitro*; Fig. 2). By 16–19 days old, follicles are at the periantral stage, and oocytes are growing and are mostly meiotically incompetent (17.2% GVB). By 21 days of age, ovaries contain antral follicles with fully grown, meiotically competent oocytes (95.4% GVB). Meiotically incompetent oocytes from 11- to 15- and 16- to 19-day-old mice were unable to promote [^{3}H]thymidine incorporation in MGC from 21- to 32-day-old mice ($P > 0.05$; Fig. 2). In contrast, coculture with competent, meiotically maturing oocytes from 21- to 32-day-old mice significantly increased MGC [^{3}H] counts above control levels ($P < 0.01$).

To investigate the possibility that the failure of meiotically incompetent oocytes to promote MGC growth is because of a different state of differentiation of the GC, an age cross-over experiment was conducted by using relatively undifferentiated GC from preantral follicles from 12- to 15-day-old mice, and more differentiated MGC from 24- to 28-day-old mice. Most notably, preantral GC had a

TABLE 1

Comparison of Coculture with Denuded Oocytes (DO) to Culture with Fresh Oocyte-Conditioned Medium (OCM) on MGC [^{3}H]thymidine Incorporation

MGC plus:	*n* oocytes/well	MGC [^{3}H] incorporation (Relative mean ± SEM)
—	0	1.00 ± 0.00[a]
OCM	8	1.35 ± 0.19[a]
DO coculture	16	5.65 ± 1.25[b]
OCM	16	1.38 ± 0.11[a]
OCM	32	1.27 ± 0.07[a]
OCM	64	1.55 ± 0.32[a]

Note. Oocyte-conditioned medium (OCM) was generated by culturing meiotically competent oocytes for 24 h at a concentration of 1.25 DO/μl. On the subsequent day, fresh OCM was diluted out to the appropriate concentrations and added to freshly collected MGC from 21- to 32-day-old mice. 64 DO/well = 0.5 DO/μl. MGC were cultured for 24 h either alone, in the presence of denuded oocytes (DO), or with OCM freshly generated from the indicated number of oocytes per well, and assessed for [^{3}H] incorporation. Means with different superscripts are significantly different ($P < 0.001$).

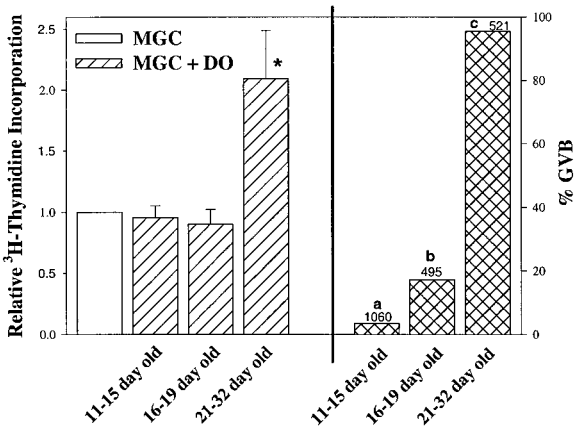

FIG. 2. Effect of meiotic competence of oocytes on DO-stimulated MGC [^{3}H]thymidine incorporation. MGC from 21- to 32-day-old mice were cultured either alone or in the presence of DO with varying degrees of meiotic competence collected from mice of increasing age prepuberty. The same oocytes were assessed for meiotic resumption (germinal vesicle breakdown, GVB) at the end of the 24-h culture (numbers above bars represent number of oocytes meiotically assessed). Oocytes from 11- to 15-day-old mice were cultured at double the concentration of the other treatments to compensate for their smaller volume. Bars represent relative mean values ± SEM expressed as a fraction of the control (MGC in the absence of DO) from five replicate experiments. *, Significantly different to all other treatments ($P < 0.01$). Bars with no common superscripts are significantly different ($P < 0.001$).

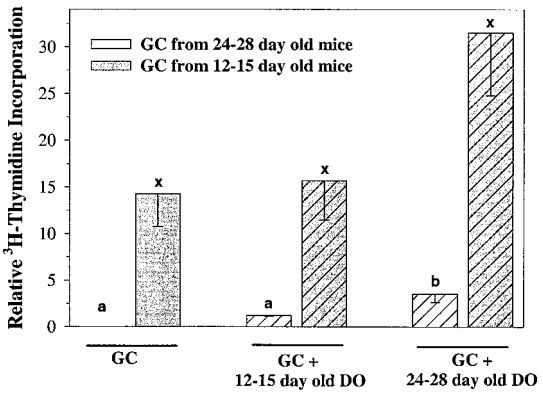

FIG. 3. Effect of state of differentiation of GC (granulosa cell) and oocyte meiotic competence on DO-stimulated GC [^{3}H]thymidine incorporation. GC from either 12- to 15-day-old or 24- to 28-day-old mice were cultured with either meiotically incompetent oocytes (from 12- to 15-day-old mice) or meiotically competent oocytes (from 24- to 28-day-old mice) and assessed for [^{3}H] incorporation after 24 h. Bars represent relative mean values ± SEM expressed as a fraction of the control (24- to 28-day-old GC in the absence of DO) from four experiments. Bars within a GC age group with no common superscripts are significantly different ($P < 0.05$).

higher base-line DNA synthetic capacity than MGC from the older prepubertal mice ($P < 0.05$; Fig. 3). Meiotically incompetent oocytes from 12- to 15-day-old mice exhibited no mitogenic activity, either on MGC or on preantral GC. By contrast, meiotically competent oocytes were able to stimulate [^{3}H]thymidine uptake by both GC types. Because oocytes from 12- to 15-day-old mice are ~1/4 the volume of fully grown oocytes and because the effects of oocytes on GC act in a dose-dependent manner, an experiment was conducted to compensate for this lower ooplasmic volume. Consistent with the previous experiment, meiotically incompetent oocytes, even with four times more oocytes than the meiotically competent group, did not increase [^{3}H]thymidine counts above control levels (Table 2). Together, these results demonstrate that the mitogenic capacity of oocytes is acquired concomitant with the acquisition of meiotic competence.

Growth-Promoting Activity of GV-Arrested Oocytes, Maturing Oocytes, and Zygotes

A comparison of mitogenic activity of meiotically competent GV-stage oocytes with *in vitro* maturing oocytes, *in vivo* matured (ovulated) oocytes, and zygotes is shown in Fig. 4. Meiotically competent oocytes stimulated GC DNA synthesis in a developmentally regulated pattern, with the highest levels in immature, GV-stage oocytes, and levels progressively declining toward the end of and after meiotic maturation. GV-stage oocytes displayed significantly higher growth-promoting activity than MII-stage oocytes and zygotes ($P < 0.01$). Meiotically competent oocytes maintained in meiotic arrest by culture with 5 μM milrinone, tended to exhibit the greatest capacity of oocytes at any developmental stage (Fig. 4). This was on average 1.5 times higher than oocytes undergoing meiotic maturation, although this difference was not significantly different (Fig. 4).

TABLE 2

Effect of Denuded Oocytes (DO) on DNA Synthesis in Granulosa Cells from 12- to 14-Day-Old Mice: Comparison of Meiotically Competent Versus Incompetent Denuded Oocytes

Meiotic competence of cocultured oocytes	*n* oocytes/well	GC [^{3}H] incorporation (Relative mean ± SEM)
—	0	1.00 ± 0.00^a
Competent	16	2.31 ± 0.25^b
Incompetent	16	1.11 ± 0.11^a
Incompetent	64	0.96 ± 0.09^a

Note. Meiotically competent oocytes were collected from 21- to 32-day-old mice, whereas incompetent oocytes were from 12- to 14-day-old mice. Granulosa cells (GC) from 12- to 14-day-old mice were cultured for 24 h with both oocyte types and assessed for [^{3}H] incorporation. Means with different superscripts are significantly different ($P < 0.001$).

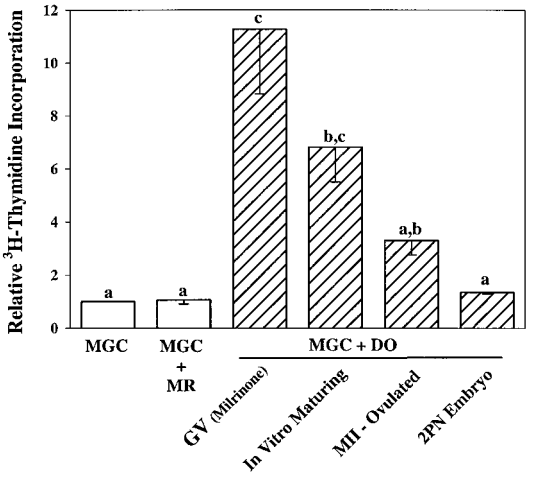

FIG. 4. Comparison of oocytes/zygotes at different stages of development on their capacity to stimulate MGC [^{3}H]thymidine incorporation. MGC from 24- to 32-day-old mice were cocultured with either milrinone-arrested germinal vesicle-stage (GV) oocytes, *in vitro* maturing oocytes, metaphase II (MII) ovulated ova, or one-cell zygotes. Bars represent relative mean values ± SEM expressed as a fraction of the control (MGC alone). Bars with no common superscripts are significantly different ($P < 0.05$).

To confirm the oocyte specificity of milrinone, GC were cultured with various concentrations of milrinone without oocytes (Fig. 5). There was no effect of milrinone ($P > 0.05$), even at concentrations as high as 125 μM, on the incorporation of thymidine into either MGC alone, or on DO-stimulated incorporation into MGC. Hence the elevated MGC [^{3}H] incorporation can be specifically attributed to the activity of GV oocytes and not due to the milrinone treatment, as no milrinone × GV–oocyte interaction was observed ($P > 0.05$; two-way ANOVA). Milrinone concentrations as low as 1 μM were sufficient to maintain 82% of oocytes at the GV stage, compared to only 12% of control oocytes (0 μM milrinone). As was also observed in Fig. 4, DO-stimulated [^{3}H] incorporation in MGC was marginally lower ($P > 0.05$) in the presence of meiotically maturing oocytes (0 μM milrinone) than with GV-arrested oocytes (1–125 μM milrinone; Fig. 5).

To examine the growth-promoting activity of oocytes during defined periods of meiotic maturation, oocytes were precultured for 0, 3, 6, or 9 h to produce four groups of oocytes, each at progressively advanced stages of meiosis. After the designated period of preculture, oocytes were then added to freshly collected MGC for just 3 h, thereby exposing MGC to oocytes at different stages of meiotic maturation (Fig. 6). The capacity of oocytes to promote MGC [^{3}H] incorporation progressively declined over the 12-h period of meiotic maturation. Oocytes undergoing GVB had the equivalent capacity to promote [^{3}H] incorporation as oocytes undergoing the diakinesis-to-metaphase I transition. The growth-promoting activity of oocytes declined most markedly in the final phase of oocyte maturation as oocytes approached metaphase II (MII; $P < 0.05$). To ensure that this decline was not an artefact of oocyte *in vitro* maturation, a comparison was made between oocytes matured *in vitro* and *in vivo* (Table 3). MII oocytes, whether matured *in vitro* or *in vivo*, had equivalent capacities to promote MGC [^{3}H] incorporation. In all three experiments, [^{3}H] counts in the presence of MII oocytes were ~50% that of meiotically maturing oocytes (Figs. 4 and 6, Table 3). Mitogenic capacity declined even further after fertilization of mature oocytes, with undetectable levels from zygotes (Fig. 4).

DISCUSSION

This study demonstrates that the growth-promoting activity of oocytes is developmentally coordinated throughout folliculogenesis and oocyte maturation. Perhaps most surprisingly, growing oocytes from preantral follicles do not appear to promote proliferation of granulosa cells, with oocytes first acquiring this capacity at the end of their growth phase. The most potent modulators of granulosa cell DNA synthesis are fully grown GV oocytes which secrete a mitogen throughout antral follicle development. Upon the resumption of meiosis, whether *in vivo* or *in vitro*, oocyte mitogenic capacity starts to fall and reaches

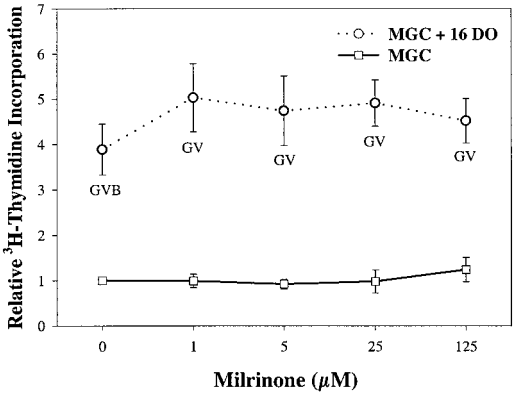

FIG. 5. Lack of interaction between milrinone treatment and GV-stage oocytes. MGC from 24- to 32-day-old mice were cultured with increasing concentrations of the type-3 phosphodiesterase inhibitor, milrinone, either alone or in the presence of 16 DO. In the absence of milrinone, denuded oocytes underwent germinal vesicle breakdown (GVB) during the culture period, whereas at all milrinone concentrations, >90% of oocytes were maintained in meiotic arrest. Points represent relative mean values ± SEM expressed as a fraction of the control (MGC in the absence of DO or milrinone). No milrinone × GV-oocyte interaction was observed (two-way ANOVA). Dose of milrinone had no effect on [^{3}H]thymidine incorporation in MGC alone or in DO-stimulated MGC. Denuded oocytes significantly increased MGC [^{3}H]thymidine incorporation above MGC alone ($P < 0.0001$).

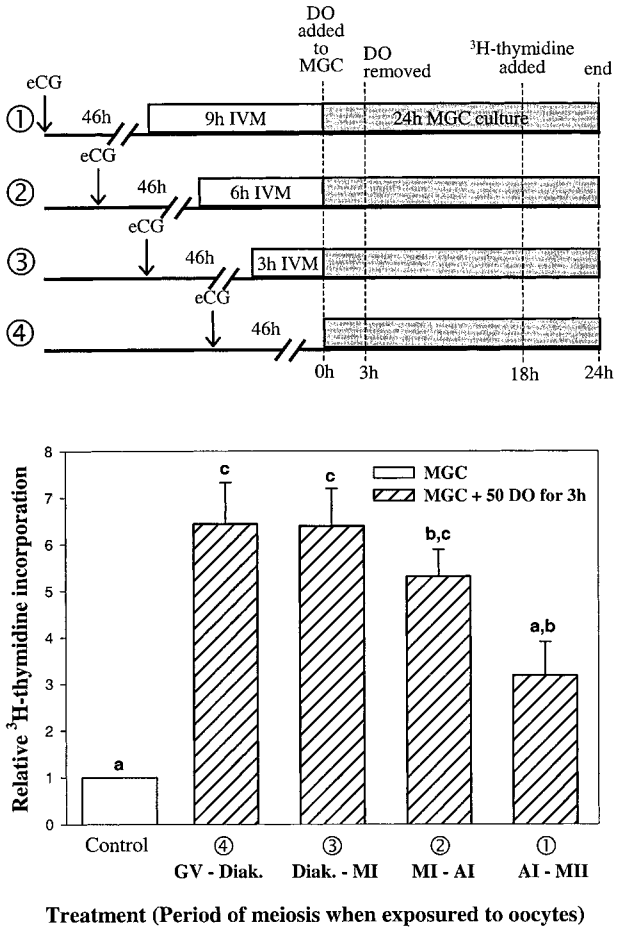

FIG. 6. Effect of stage of meiosis on the capacity of DO to stimulate MGC [^{3}H]thymidine incorporation. MGC from 24- to 28-day-old mice were exposed to denuded oocytes for 3 h at defined stages of meiotic maturation; either oocytes undergoing germinal vesicle breakdown, reaching MI, undergoing the MI-AI transition, or completing meiotic maturation. To achieve this, groups of 50 oocytes were precultured for either 9 h (treatment 1), 6 h (treatment 2), 3 h (treatment 3), or 0 h (treatment 4), then added to fresh MGC for just 3 h before being removed from wells. The remaining MGC were cultured for a further 21 h and then assessed for [^{3}H] incorporation. Bars represent relative mean values ± SEM expressed as a fraction of the control (MGC in the absence of DO). Bars with no common superscripts are significantly different ($P < 0.05$).

base-line levels after fertilisation. This expression profile is somewhat unusual because the developmental pattern of oocyte mitogenic activity is the converse of oocyte transcriptional activity. For example, growing oocytes and meiotically maturing oocytes have higher rates of transcription than fully grown GV-arrested oocytes (de Smedt *et al.*, 1994; Fair *et al.*, 1995). GV oocytes in antral follicles are at a relatively quiescent phase of development as indicated by condensed chromatin configurations and compact nucleoli (Crozet, 1989; Mattson and Albertini, 1990). Yet in this study, it is precisely these oocytes that exhibit the highest mitogenic activity, indicating that the growth-promoting activity of oocytes is differentially regulated from general oocyte synthetic activity.

This study has shown that growing, meiotically incompetent oocytes are unable to promote the proliferation of granulosa cells from either preantral or antral follicles. Both granulosa cell types are, however, responsive to an oocyte mitogen as evidenced by promotion of GC thymidine incorporation by meiotically competent oocytes. This suggests that the oocyte may not be a major promoter of the growth of preantral granulosa cells, and that perhaps nonoocyte paracrine factors are driving the earlier stages of folliculogenesis. Oocytes from preantral follicles do, however, contain GDF-9 (Elvin *et al.*, 1999; Jaatinen *et al.*, 1999) and BMP-15 proteins (Otsuka *et al.*, 2000), both of which are potent GC mitogens (Otsuka *et al.*, 2000; Vitt *et al.*, 2000a). Furthermore, oocyte GDF-9 and BMP-15 are prerequisites for follicle growth beyond the primary stage in mice (Dong *et al.*, 1996) and sheep (Galloway *et al.*, 2000), respectively. GDF-9 also stimulates rodent preantral follicle growth both *in vitro* (Hayashi *et al.*, 1999) and *in vivo* (Vitt *et al.*, 2000b). These studies demonstrate that preantral follicles are responsive to GDF-9 and BMP-15 and that oocyte-secreted GDF-9 and BMP-15 are key regulators of early folliculogenesis.

However, results of the present and of other studies indicate that activities of oocyte-secreted factors cannot be fully attributed to GDF-9 and BMP-15 activities. For example, recombinant GDF-9 suppresses KL mRNA expression in both preantral GC and MGC, yet oocytes from preantral follicles are not able to suppress KL levels, in contrast to fully grown oocytes from antral follicles (Joyce *et al.*, 2000). Similarly, GC mucification is promoted and uPA expression is suppressed by GDF-9 and fully grown oocytes, but not by growing oocytes (Vanderhyden *et al.*, 1990; Canipari *et al.*, 1995; Elvin *et al.*, 1999). These findings parallel the situation with the oocyte mitogen: GC

TABLE 3

Mitogenic Capacity of *in Vivo* Versus *in Vitro* Matured Oocytes

Nuclear status of cocultured oocytes	MGC [^{3}H] incorporation (Relative mean ± SEM)
—	1.00 ± 0.00^a
In vitro maturing	16.15 ± 2.08^b
MII—*in vitro* matured	9.15 ± 0.59^c
MII—ovulated	7.69 ± 0.82^c

Note. MGC from 21- to 32-day-old mice were cultured either alone, with oocytes undergoing meiotic maturation (freshly isolated at the GV-stage), or with meiotically mature oocytes either *in vivo* or *in vitro* matured in the previous 12 h. Means with different superscripts are significantly different ($P < 0.01$).

proliferation is promoted by GDF-9, BMP-15, and fully grown oocytes (Vanderhyden *et al.*, 1992; Otsuka *et al.*, 2000; Vitt *et al.*, 2000a), but not by growing meiotically incompetent oocytes (present study). Therefore, although GC from preantral follicles are responsive to GDF-9, BMP-15, and factors from fully grown oocytes, in general, oocytes from preantral follicles do not elicit a GC response. If the effects of fully grown oocytes are mediated through GDF-9, BMP-15 or a heterodimer of the two, then the GDF-9 and BMP-15 present in growing oocytes must have limited biological activity, at least in terms of mitogenic activity, promoting mucification, or suppressing uPA levels. Although GDF-9 and BMP-15 proteins have been detected in oocytes by using immunohistochemistry, thus far there is no report of actual secretion of biologically active GDF-9 or BMP-15 by oocytes. Perhaps the GDF-9 and BMP-15 proteins detected in growing oocytes are not actively secreted, or are secreted in their biologically inactive preproprotein forms. Clearly GDF-9 and BMP-15 are critical for follicle transition beyond the primary stage, but their roles in preantral follicle growth may require further clarification.

In this study, oocytes with the highest mitogenic activity were fully grown, GV-stage oocytes. Meiotic resumption of competent oocytes was artificially inhibited by treatment with milrinone, a selective inhibitor of type-3 cyclic nucleotide phosphodiesterases. These experiments exploited the fact that different cell types within rodent follicles contain different phosphodiesterase isotypes; that is, oocytes contain type-3B phosphodiesterase while follicular somatic cells contain type 4D (Tsafriri *et al.*, 1996). When studying oocyte–follicular cell interactions, inhibiting meiosis using an oocyte-specific type-3 phosphodiesterase inhibitor has the advantage over other commonly used noncell-type-specific agents such as 3-isobutyl methylxanthine (IBMX) or cycloheximide, as the function of follicular cells is not effected by the meiotic inhibitor.

The growth-promoting activity of oocytes is attained once oocytes have completed their growth phase and have acquired meiotic competence. The mitogenic activity of oocytes was higher in fully grown, GV-arrested oocytes than in oocytes from any other stage of development. This is noteworthy because, in general terms, this is a relatively quiescent stage of oogenesis. At this stage of development, oocytes have completed their growth phase and rates of transcription are notably less than in growing oocytes or meiotically maturing oocytes. This stage of oogenesis persists approximately from early antrum formation through antral development up to the preovulatory-stage of follicle development. During this stage, both cumulus cells and MGC are able to respond to the oocyte mitogen (Li *et al.*, 2000; present study); however, presumably cumulus cells are the primary recipients. This concept is supported by the fact that bovine COC have a much higher proliferative capacity than MGC, and cumulus cells are more sensitive than MGC to the oocyte mitogen (Li *et al.*, 2000). Furthermore, radiolabelling in antral follicles of rats infused with [^{3}H]thymidine is highest in the COC and the surrounding MGC, and lowest in MGC on the opposite side of the follicle (Hirshfield, 1986). These data suggest that a morphogenic gradient of oocyte-secreted factors exists across the antral follicle and that in general, MGC are either not exposed to the paracrine actions of oocytes or have some mechanism to neutralise their effects. These ideas are in keeping with the hypothesis that the default pathway of follicular cell development is towards the MGC phenotype and that the oocyte actively promotes development of the cumulus cell phenotype (Eppig *et al.*, 1997; Li *et al.*, 2000). Maintenance of a specialised oocyte–cumulus cell microenvironment throughout antral development by oocyte factors may constitute an important component of oocyte cytoplasmic maturation and hence oocyte developmental capacity.

Meiotic resumption marks the beginning of the end of secretion of a growth-promoting factor by the oocyte. After meiotic resumption, oocytes continue to secrete a granulosa cell mitogen at roughly constant levels for the first 6 h and then activity progressively declines to undetectable levels by the zygotic stage. Hence oocytes are actively secreting a mitogen during a critical phase of oocyte development, when the oocyte is undergoing dramatic changes in its cell program, including meiotic maturation and remodelling of the surrounding cumulus investments. Other oocyte-regulated activities such as steroid secretion, and suppression of uPA and LHR mRNA expression are all maintained to some extent by metaphase II oocytes, but are then absent in the early zygote (Canipari *et al.*, 1995; Eppig *et al.*, 1997; Vanderhyden and Macdonald, 1998). The developmental expression of oocyte CEEF is also tightly regulated during meiotic maturation. Fully grown mouse oocytes secrete CEEF when arrested at the GV stage and during meiotic maturation, but not after fertilization (Vanderhyden *et al.*, 1990). It seems reasonable that the continued production of all of these oocyte factors during oocyte maturation is important for remodelling of the cumulus investments in preparation for ovulation and fertilization. This includes precise regulation of cumulus cell steroidogenesis, expansion of the cumulus cell mass and withdrawal of the oocyte–cumulus cell cytoplasmic processes, and maintenance of extracellular-matrix stability by inhibiting local protease activity. However, it is less clear what the function of an oocyte-secreted mitogen might be during meiotic maturation, as opposed to during antral development, when the oocyte-mitogen is required to maintain the microenvironment of the oocyte–cumulus cell complex distinct from that of the MGC (Li *et al.*, 2000). Perhaps secretion of the oocyte-mitogen during meiosis serves some, as yet undetermined role, or alternatively secretion during meiotic maturation may just represent residual activity from when the oocyte was arrested at the GV stage. This notion is supported by the fact that soon after meiotic resumption, oocyte mitogen levels progressively decline during and after oocyte maturation.

This study has shown that the capacity of mouse oocytes to promote growth of granulosa cells is regulated in a

developmentally coordinated manner. The failure of meiotically incompetent oocytes to promote growth is inconsistent with the predicted role of oocyte-secreted GDF-9 and BMP-15 in early folliculogenesis. The developmental expression pattern of the oocyte mitogen is however consistent with many other oocyte-secreted factors, with activities being first attained as oocytes acquire meiotic capacity, maintaining high expression levels throughout antral development, and then falling after meiotic maturation. This expression profile adds further support to the growing body of evidence that oocytes secrete these paracrine factors from around the time of antrum formation until ovulation, to establish and maintain an immediate microenvironment which is distinct from that of the rest of the follicle.

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